

Clomiphene citrate challenge test and serum anti-Müllerian hormone levels in women with menstrual irregularities and/or infertility

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Abstract: Aim: Evaluation of CCCT results and anti-Müllerian hormone (AMH) plasma levels in women with menstrual cycle irregularities and/or infertility. Patients and methods: 70 patients with menstrual cycle disturbances were recruited to the study. Clomiphene citrate challenge test (CCCT) was carried out in each patient enrolled. At day 3 of the cycle plasma basic levels of FSH, estradiol, progesterone, IGFBP-1, TSH, prolactin, DHEAS and anti-Müllerian hormone were measured. At day 10 of the cycle plasma levels of FSH and estradiol were evaluated. Plasma levels of hormones were measured by commercially available ELISA kits. Results: 50 women with normal result of CCCT (group A) had higher mean plasma level of AMH compared to 20 women with abnormal result of CCCT (group B). Mean plasma FSH level at day 3 of the cycle was lower in group A compared to group B. There were no other statistically significant differences in mean values of assessed parameters between groups A and B. Taking into account all patients enrolled to the study AMH correlated significantly with patients' age and plasma levels of FSH at day 3 and day 10 of the cycle). Basic AMH plasma levels in group A correlated negatively with plasma levels of FSH at day 3 and day 10. In group B plasma levels of FSH at day 10 of the cycle also correlated with basic AMH plasma levels. Plasma levels of estradiol at day 10 of the cycle were related inversely with basic AMH plasma levels in group A, but directly in group B. Conclusion: It should be recommended to perform the CCCT before infertility treatment. Evaluation of the anti-Müllerian hormone plasma level reflects the results of the CCCT.

Key words: Clomiphene citrate - Challenge test - Anti-Müllerian hormone - Women - Menstrual irregularities - Infertility

Introduction

Ovarian reserve comprises two elements: the size of the stock of primordial follicles and the quality of the oocytes [1]. Ovarian reserve may be assessed by static tests such as: measure of early follicular phase basal FSH, inhibin B, morphometric ultrasonographic measures such as antral follicle count or ovarian volume [2]. Dynamic tests of ovarian reserve include clomiphene citrate challenge test (CCCT) and the GnRH agonist stimulation test [2]. Eldar-Geva *et al.* found that dynamic endocrine tests were better than

basal evaluation of FSH, inhibin B or estradiol in predicting ovarian reserve [2]. Kahraman *et al.* showed that abnormal CCCT result is a good predictor of diminished ovarian reserve and this dynamic test is better than such static test as basal FSH concentration on day 3. It provides valuable information for both patients as to their chances of achieving a pregnancy and also for the medical team deciding on options for stimulation protocols [3]. Csemiczky *et al.* found that predictive value of the CCCT for a negative outcome of IVF treatment was strong and recommended performing the test before infertility treatment [4]. Van Swieten EC *et al.* suggested that CCCT was a good predictor of IVF and IVF/ICSI outcome [5]. Women with an abnormal CCT needed more days of stimulation and higher doses of gonadotropins to reach an adequate stimulation than women with a normal CCCT [5].

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Table 1. The clinical characteristics of the study group.

Parametr	Mean value n=70
Age (years)	29.9±5.5
Body mass (kg)	60.7±14.7
TSH (IU/l)	2.3±1.01
PRL (ug/l)	17.3±9.8
DHEAS (ug/dl)	493.3±239.2
IGFBP-1 (ug/l)	26.7±16.9
Testosterone (nmol/l)	2.4±1.4
AMH (ug/l)	6.2±6.9
FSH 3 rd dc (IU/l)	9.1±11.8
FSH 10 th dc (IU/l)	11.8±11.8
Estradiol 3 rd dc (ng/l)	71.5±66.3
Estradiol 10 th dc (ng/l)	426.8±337.7
Progesterone 3 rd dc (ug/l)	1.27±1.84

The dimeric glycoprotein anti-Müllerian hormone (AMH) is a member of the transforming growth factor- β (TGF- β) family. It was identified as a factor produced by Sertoli cells, which causes regression of the Müllerian ducts during male fetal development [6]. Expression of AMH mRNA was also found in the human granulosa cells of the primordial follicles immediately after their formation and subsequently in granulosa cells of secondary preantral stage follicles and small antral follicles [7]. AMH protein expression is detected in the granulosa cells up to the moment when the follicles gain FSH dependence [8]. Durlinger *et al.* suggested that AMH might be involved in the recruitment of FSH sensitive follicles in the early antral stage [9]. AMH is detected in measurable amounts in human female serum during reproductive life span [10]. AMH is associated with ovarian response in IVF patients with normal FSH levels [11].

Therefore it seemed interesting to evaluate the plasma levels of AMH levels in women menstrual cycle irregularities with normal and abnormal results of CCCT test.

Materials and methods

Patients. 70 patients with menstrual cycle disturbances were recruited to the study. The study was approved by the Jagiellonian University Bioethical Committee. The clinical characteristics of the group are shown in Table 1. Each patient had the basal hormonal evaluation (FSH, estradiol, TSH, prolactin, DHEAS, progesterone, IGFBP-1) carried out at day 3 of the cycle.

Clomiphene citrate challenge test. Clomiphene citrate challenge test (CCCT) was carried out in each patient enrolled. At day 3 of the cycle plasma basic levels of FSH, estradiol, progesterone, TSH, prolactin, DHEAS, IGFBP-1 and anti-Müllerian hormone were

Table 2. The comparison of clinical characteristics and hormonal results between group A (patients with normal CCCT result) and group B (patients with abnormal CCCT result).

Parametr	Group A n=50	Group B n=20	p
Age (years)	30.6±6.0	30.1±5.5	NS
Body mass (kg)	62.1±11.2	58.5±10.2	NS
TSH IU/l)	2.0±0.8	2.3±0.9	NS
PRL (ug/l)	19.0±8.9	17.0±8.5	NS
DHEAS (ug/dl)	508.8±233.0	471.8±171.0	NS
IGFBP-1 (ug/l)	25.5±16.5	27.5±15.8	NS
Testosterone (nmol/l)	2.6±1.7	2.3±0.9	NS
AMH (ug/l)	6.95±7.8	1.45±1.5	<0.02
FSH 3 rd dc (IU/l)	7.0±1.4	13.2±5.5	<0.05
FSH 10 th dc (IU/l)	11.2±11.0	18.4±17.3	NS
Estradiol 3 rd dc (ng/l)	61.1±26.2	70.0±58.4	NS
Estradiol 10 th dc (ng/l)	468.4±349.2	568.0±321.8	NS
Progesterone 3 rd dc (ug/l)	0.8±0.7	1.1±0.5	NS

measured. At day 10 of the cycle plasma levels of FSH and estradiol were evaluated. CCCT consisted of 5 days of clomiphene citrate oral administration by 100 mg daily starting from day 3 of the cycle. All sample tubes were kept and transported in the ice box, than centrifuged as soon as possible at 4°C (refrigerated centrifuge, Heraeus, Germany). Plasma was stored frozen at -70°C until the determination of the hormones, not later then after 3 months of storage. Frozen samples were thawed slowly only once and incubated for 30 minutes at 4°C prior the analysis. FSH and estradiol levels were measured using ECLIA Roche kits (sensitivity: 1 ng/ml for estradiol, cross reaction with LH, TSH, human chorionic gonadotropin 0.039%; sensitivity for FSH of 0.5 mIU/ml less than 5% interference with bilirubin and haemoglobin, cross-reaction with hCG 0.016%). IGFBP-1 level was measured by ELISA (Diagnostic System Laboratories, USA - DSL-10-7800 Active): sensitivity 0.25 ng/ml; the inter- and intra-assay coefficients of variation were 7.6% and 4.6%, respectively; no cross reactivity with IGF-I, IGF-II, insulin, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6. Anti-Müllerian hormone levels were measured by ELISA (Diagnostic System Laboratories, USA - DSL-10-14400 Active). Sensitivity 0.006 ng/ml; the inter- and intra-assay coefficients of variation were 8.0% and 4.6%, respectively. PRL, TSH, Testosterone, DHEAS, progesterone were measured by ECLIA Roche kits.

Statistical analysis. Descriptive statistics, including mean values, median and SE, were calculated for all the variables. The data obtained for women in each group were compared by the paired t-test. For comparisons between the groups, a non-parametric test was applied. Significance level was set at $p < 0.05$. Statistical calculations were performed using Statistica 3.1 software.

Results

50 women with normal result of CCCT (group A) had higher mean plasma level of AMH compared to 20 women with abnormal result of CCCT (group B)

($6.95 \pm 7.8 \mu\text{g/l}$ vs $1.45 \pm 1.5 \mu\text{g/l}$ $p < 0.02$). Mean plasma FSH level at day 3 of the cycle was lower in group A compared to group B ($7.0 \pm 1.4 \text{ IU/l}$ vs $13.2 \pm 5.5 \text{ IU/l}$; $p < 0.05$). There were no other statistically significant differences in mean values of assessed parameters between groups A and B (Table 2).

Taking into account all patients enrolled to the study AMH correlated significantly with patients' age ($r = -0.41$; $p < 0.05$) and plasma levels of FSH at day 3 and day 10 of the cycle ($r = -0.34$; $p < 0.05$; $r = -0.35$; $p < 0.05$ resp.). Basic AMH plasma levels in group A correlated negatively with plasma levels of FSH at day 3 and day 10 ($r = -0.40$; $p < 0.05$; $r = -0.35$; $p < 0.05$ resp.). In group B plasma levels of FSH at day 10 of the cycle also correlated with basic AMH plasma levels ($r = -0.56$; $p < 0.05$). Plasma levels of estradiol at day 10 of the cycle were related inversely with basic AMH plasma levels in group A ($r = -0.40$; $p < 0.05$), but directly in group B ($r = 0.64$; $p < 0.05$).

Discussion

Our results indirectly showed that basic plasma levels of anti-Müllerian hormone measured at day 3 of the cycle may be applied as one of markers of ovarian reserve. We showed that women with diminished ovarian reserve (abnormal CCCT result, higher FSH plasma level) had also lower, basic plasma level of AMH despite the lack of differences in mean age. Te Vet *et al.* showed the age related decline of AMH plasma level [1]. AMH is solely produced by the granulosa cells of growing follicles and therefore reflects the quantity and quality of ovarian follicle pool [1]. Rooij *et al.* showed the correlation between the plasma levels of AMH and the number of antral follicles and the number of oocytes retrieved [12]. Eldar-Geva *et al.* suggested that basic follicular plasma level of AMH is the only predictor for achieving pregnancy in IVF stimulated cycles [1].

We also showed that women with abnormal CCCT result had higher FSH plasma levels during the CCCT (day 3 and day 10). Csemiczky *et al.* showed similar data regarding the link between abnormal results of CCCT and plasma FSH level [4].

Fried *et al.* found that IGF-I/IGFBP-1 ratio might have reflected oocyte quality. IGF-I/IGFBP-1 ratio in serum as well as in follicular fluid was significantly higher in women who became pregnant [13]. In our study we observed a tendency toward lower IGFBP-1 serum concentration in women with normal CCCT.

That may correspond to the higher IGF/IGFBP-1 ratio observed by Fried *et al.* To conclude, it should be recommended to perform the CCCT before infertility treatment. Evaluation of the anti-Müllerian hormone plasma level reflects the results of the CCCT.

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